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Kinetics of in vitro adsorption and entry of papillomavirus virions

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Abstract

There has been much incongruence in reports addressing the rate at which papillomaviruses enter cultured cells. We used a recently developed QRT-PCR assay (J. Virol. Methods 111 (2003) 135) to analyze the expression, adsorption, and entry kinetics of human papillomavirus type 11 (HPV-11) in multiple cell lines. Parallel experiments with HPV-40 and cottontail rabbit papillomavirus (CRPV) were also performed with biologically relevant lines. Infection was determined by the expression of early transcripts containing the E1^E4 splice junction. Results support previous observations that papillomaviruses may enter cultured cells much more slowly than rates reported for similarly structured viruses (Virology 207 (1995) 136; Virology 307 (2003) 1; J. Virol. 75 (2001) 1565). Additionally, our data suggest that, following adsorption to the cell surface, capsomeric structure remains largely unchanged for many hours as HPV-11 virions remain equally susceptible to neutralization by a nonspecific microbicide and by L1-specific monoclonal antibodies (MAb) targeting both linear and conformationally sensitive epitopes.

Keywords: Papillomavirus; HPV; Viral entry; QRT-PCR; Real-time RT-PCR; Virus neutralization; Virus adsorption; Entry kinetics

Introduction

Papillomaviruses comprise a large family of naked DNA tumor viruses that infects the cutaneous or mucosal epithelia of dozens of vertebrate species with a strict species tropism. Over 100 papillomavirus types have been identified in human tissues, most targeting cutaneous epithelium with the remainder infecting mucosal surfaces. The latter are considered the etiological agents of cervical cancer and warts of the genital and respiratory tracts. Papillomaviruses replicate only in terminally differentiating epithelium (Stubenrauch and Laimins, 1999). Successful infections occur when virions gain access to the basal layer of stratified epithelia. As infected cells move more distant from the basement membrane, the transcription of viral DNA (vDNA) is altered so that the late genes encoding for the two capsid proteins, L1 and L2, are expressed only in the uppermost terminally differentiated epithelial cells (Meyers et al., 1992). Mature virions are presumably spread via the shedding squames.

Nonpermissive infection of cultured cells by papillomaviruses has been used to model the early events in a natural infection. Authentic virions have been obtained from natural lesions (Crawford and Crawford, 1963), from infected xenografts implanted into immunocompromised mice (Bonnez et al., 1993; Brown et al., 1998; Kreider et al., 1987), and from organotypic "raft" culture systems (Meyers et al., 1992, 1997; Ozbun, 2002). Infection of cells cultured in monolayer has been used to demonstrate that papillomaviruses can adsorb to multiple human, mammalian, and even insect cell lines (Müller et al., 1995), traffic to the perinuclear zone in a cytoskeleton-dependent manner (Liu et al., 2001; Zhou et al., 1995), generate multiple viral transcripts (Chow et al., 1987), and replicate the viral genome (LaPorta and Taichman, 1982). Due to the difficulty in obtaining authentic infectious papillomavirus virions, the events of early infection have also been modeled using surrogate virus particles such as empty capsids termed "virus-like particles" (VLPs) and capsids carrying reporter plasmids termed "pseudovirions" (Bousarghin et al., 2003; Joyce et al., 1999; Kirnbauer et al., 1992; Müller et al., 1995; Roden et al., 1994; Sapp et al., 1995).

Experiments reporting on the entry (i.e. internalization) kinetics of papillomavirus virions, or surrogate particles, into

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cultured cells have yielded conflicting results. Early experiments utilized bovine papillomavirus type 1 (BPV-1) applied to monolayers of CV-1 cells (green monkey kidney) or C127 cells (mouse mammary tumor) at very high multiplicities of infection (m.o.i.) (Liu et al., 2001; Zhou et al., 1995). Electron microscopy (EM) was used to determine the location of virions over a time course postinfection (p.i.). Images revealed that the majority of virions was endocytosed within 30 min and co-localized with LAMP-1 (Zhou et al., 1995). Large numbers of virus particles were shown to accumulate in the perinuclear zone within 60 min (Liu et al., 2001). Additional evidence suggesting the rapid entry of papillomaviruses into cultured cells came from the discovery of viral transcripts in BPV-4-infected bovine fetal palate keratinocytes at 2 h p.i. (Sibbet et al., 2000) and in HPV-31b-infected HaCaT cells (spontaneous immortalized human keratinocytes) at 4 h p.i. (Ozbun, 2002). In both cases, cultured cells were infected at relatively low m.o.i. and viral transcripts were detected by reverse transcription followed by two sequential rounds of polymerase chain reaction using nested primers (nested RT-PCR).

Evidence that papillomaviruses enter cultured cells with much slower kinetics was seen in the ability of capsid-targeting neutralizing antibodies to reduce the end-point measure of infection even when applied many hours post-infection. We previously reported that the number of BPV-1-induced foci formed in monolayers of C127 cells could be reduced 2-fold or more by the addition of capsid-targeting antibodies to cell culture supernatants 24 h p.i. (Christensen et al., 1995). BPV-1 was later reported to be taken up by C127 cells with a $t_{1/2}$ of 4 h via a clathrin-dependent pathway (Day et al., 2003). Additionally, infection of A431 cells (human epithelial carcinoma) by human papillomavirus type 11 (HPV-11) virions could be completely blocked by a nonspecific high-molecular-weight microbicide added to cell supernatants even 4 h p.i. as assayed by a sensitive nested RT-PCR assay (Christensen et al., 2001). Similar experiments performed with HPV-33 pseudovirions have shown that the successful delivery of a GFP-reporter plasmid could be completely blocked by an L1-specific monoclonal antibody (MAb) applied 4 h after attachment of particles to COS-7 cells (green monkey kidney cells transformed with SV40) (Giroglou et al., 2001). The ability of capsid-targeting MAb to neutralize the bulk of applied papillomaviruses many hours postinfection appears incongruent with the rapid internalization reported by EM-based studies (Liu et al., 2001; Zhou et al., 1995) and with the entry kinetics reported for other small, naked, DNA viruses such as SV-40 and mouse polyomavirus (An et al., 2000; Clever et al., 1991; Kartenbeck et al., 1989; Mackay and Consigli, 1976).

We used a recently described quantitative RT-PCR (QRT-PCR) assay (Culp and Christensen, 2003) to analyze the kinetics of papillomavirus infection. Infection studies were performed in multiple cell lines to determine the kinetics of viral gene expression, adsorption, and escape from neutralization. The majority of described experiments utilized HPV-11 virions; however, we also present evidence that cottontail rabbit papillomavirus (CRPV) and HPV-40 virions have similar infection kinetics. Our results suggest that papillomaviruses may enter cultured cells very slowly with a large fraction of infectious particles remaining both accessible and susceptible to neutralization by MAb for >24 h. During this apparent delay in entry, capsomeric structure appears to be largely maintained as virions remain equally susceptible to nonspecific microbicides and to MAbs binding linear or conformationally sensitive surface epitopes.

Results

HPV-11 E1^E4 expression profiles

Our first set of experiments was designed to determine the pattern and reproducibility of the expression of viral E1^E4 transcripts during a time course following infection with HPV-11 virions. Transcripts containing the E1^E4 splice junction are prevalent following HPV-11 infection (Stoler et al., 1990) with E1^E4 transcript levels correlating linearly with viral input (Culp and Christensen, 2003). Near confluent monolayers were infected at a multiplicity of 150 (virus particles per cell). At the indicated time points postinfection, the cell contents were harvested and assayed for viral transcripts (Fig. 1). In each case, the expression of HPV-11 E1^E4 transcripts over the time course shows a high degree of reproducibility. Importantly, distinct profiles indicate that the delivery and expression of vDNA is characteristic for individual cell lines. Three cell lines tested, A431, HaCaT, and KH-SV (human keratinocytes transformed by SV40), have similar profiles with the levels of E1^E4 transcripts increasing throughout the time course. While comparisons between different cell lines and experiments should be made with caution, the absolute Ct values for E1^E4 transcripts consistently indicated that HPV-11 expression in KH-SV cells was many fold less than expression in A431, HaCaT, or COS-7 cells (Table 1). Like the KH-SV cells, the BO-SV cells (human keratinocytes transformed by SV40) also expressed low levels of E1^E4 transcripts following infection with HPV-11. Despite relatively poor expression, however, BO-SV cells yielded a unique and reproducible saddle-shaped expression profile.

COS-7 cells yielded a distinct expression profile for HPV-11 E1^E4. The QRT-PCR assay revealed the highest levels of viral transcripts accumulated in COS-7 at 48 h p.i., and unlike the other cell lines tested, the COS-7 cells did not sustain or increase in E1^E4 levels after this time point, but rather showed a sharp decline in viral transcripts. We saw no change in COS-7 morphology, total RNA levels, or endogenous reference transcript levels (TBP) coincidental with the drop in E1^E4 levels (data not shown).

Fig. 1 suggests that, in general, the number of viral transcripts in infected cells increases slowly and gradually

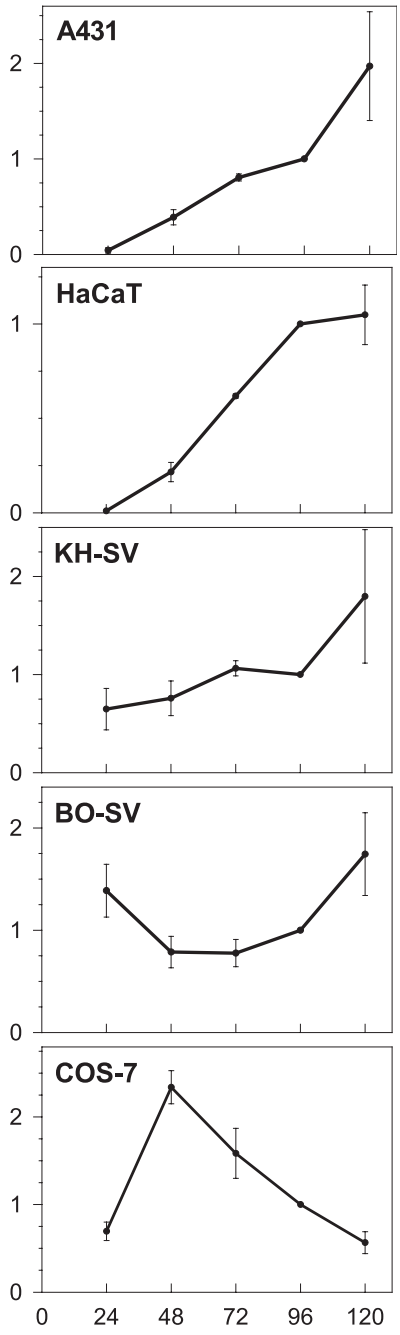

Fig. 1. Expression profiles of HPV-11. Near confluent monolayers were infected with HPV-11 at an m.o.i. of 150 particles per cell in 1 ml of medium followed by incubation at 37 °C. Twenty-four hours postinfection, monolayers were fed an additional 2 ml of medium. Monolayers were harvested with TRIzol at the indicated hours postinfection (abscissa). Expression of E1^E4 transcripts at indicated time points was determined by QRT-PCR and is shown relative to expression at 96 h (ordinate). Results shown are means (±SEM) for two independent experiments for each cell line.

in the days following infection. Possible explanations for this gradual increase in E1^E4 transcripts include slow adsorption kinetics, slow entry kinetics, and a delay in expression following delivery of the vDNA.

Limited early E1^E4 expression

Except for the BO-SV cells, the expression profiles (Fig. 1) for all cell lines tested show a pattern of slowly accumulating E1^E4 transcripts in the RNAs of infected cells for 48 h or more following infection. To evaluate the expression of viral transcripts at earlier time points, we performed the second set of expression experiments for HaCaT and COS-7 cells with additional RNA harvests at 6 and 12 h p.i. (Fig. 2A). These two cell lines were chosen due to their superior expression of E1^E4 transcripts as judged by raw Ct values (Table 1). In HPV-11-infected HaCaT cells, the expression of viral transcripts was undetectable by QRT-PCR at 6 h and was barely detectable at 12 h p.i. COS-7 cells had measurable, but very low relative E1^E4 levels at both of these early time points.

We have previously shown that the QRT-PCR assay is more sensitive than RT followed by a single round of PCR, but less sensitive than nested RT-PCR as judged by visualization of amplicons in ethidium-stained agarose (Culp and Christensen, 2003). To determine if E1^E4 transcripts could be detected at the early time points in both cell types, we used nested RT-PCR to analyze select RNA isolates previously assayed by QRT-PCR (Fig. 2B). Results of nested RT-PCR demonstrate that at 6 h p.i., the isolated RNAs from both COS-7 and HaCaTs contain detectable E1^E4 transcripts. These results confirm that at least a few virions can enter cultured cells rapidly and are consistent with earlier reports that nested RT-PCR can detect early BPV-4 and HPV-31b transcripts at 2 and 4 h p.i., respectively (Ozbun, 2002; Sibbet et al., 2000). The presence of viral transcripts containing the E1^E4 splice junction at 6 h p.i. is also congruent with earlier reports that these transcripts are the most prevalent during HPV-11 infection (Stoler et al., 1990). The targeted E1^E4 transcripts are demonstrated here to be expressed soon after infection and thus are appropriate for this investigation.

While E1^E4 transcripts can be detected by nested RT-PCR in at least two cell lines at only 6 h p.i., the QRT-PCR results clearly demonstrate that the bulk of these transcripts

Table 1
Mean Ct values (±SEM) for peak E1^E4 expression

Cell line	Peak E1^E4 expression (h p.i.)	E1^E4 Ct value	TBP Ct value
A431	120	28.84 ± 0.04	21.09 ± 0.03
HaCaT	120	27.93 ± 0.04	20.68 ± 0.10
KH-SV	120	31.47 ± 0.15	24.37 ± 0.04
BO-SV	120	32.98 ± 0.21	23.77 ± 0.15
COS-7	48	27.30 ± 0.08	19.98 ± 0.06

Values are calculated from triplicate multiplex reactions. Ct values for the endogenous reference transcript (TATA-binding protein, TBP) in KH-SV and BO-SV are higher than for other cell lines due to primer limitation required to keep E1^E4 Ct values as low as possible (Culp and Christensen, 2003).

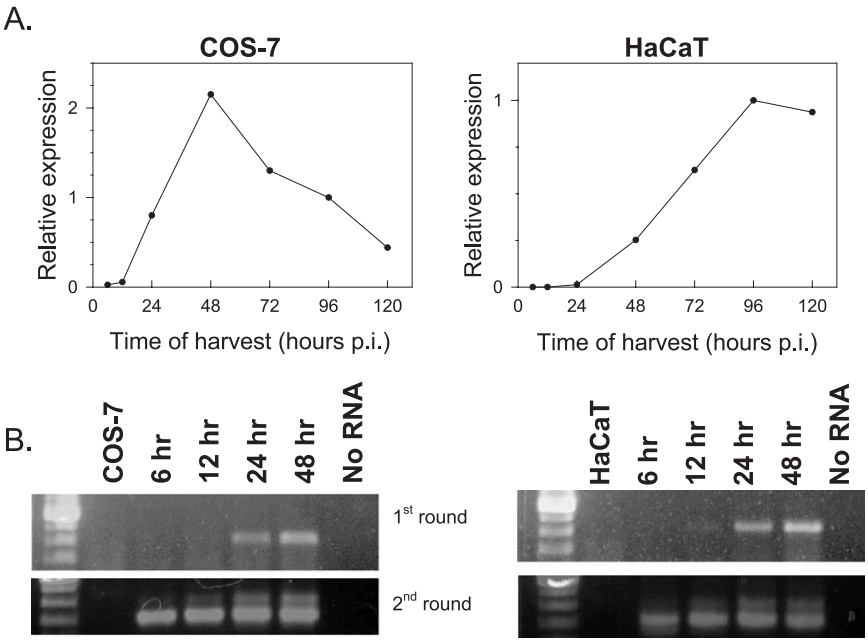

Fig. 2. Detection of E1^E4 transcripts at earlier time points. Monolayers of COS-7 and HaCaTs were infected as described in Fig. 1, with additional harvests at 6 and 12 h p.i. Levels of viral transcripts in total isolated RNAs were assayed by both QRT-PCR (A) and nested RT-PCR (B).

is not present in the isolated RNAs of infected cells until many hours later (Fig. 2A).

HPV-40 and CRPV expression

To determine if the delayed expression of viral transcripts was virus type-specific or representative of other papillomaviruses, we infected A431, HaCaT, and COS-7 cells with HPV-40 virions (undetermined m.o.i.). As shown in Fig. 3, the level of HPV-40 E1^E4 transcripts peaked in both HaCaT and COS-7 cells at 72 h p.i. Interestingly, while the COS-7 cells displayed similar expression profiles for both HPV-11 and -40, the HaCaT cells did not maintain high levels of E1^E4 transcripts following infection with HPV-40 as they did reproducibly with HPV-11. Rather, the HaCaT expression profile mirrors that of the COS-7 cells. A431 cells showed similar expression profiles for both HPV-11 and HPV-40 E1^E4 with peak expression at 120 h p.i. We repeated the HPV-40 infection experiments using a 10-fold reduction in virus input and saw similar results for all three cell types, suggesting that the difference in the expression of HPV-11 and HPV-40 by HaCaT cells is due to a type-specific virus–host cell interaction and is not an artifact of a higher virus input (data not shown).

To further explore the infection kinetics of papillomaviruses we infected RK13 cells (rabbit keratinocytes) with authentic CRPV virions (m.o.i. unknown) (Fig. 3). Following infection with CRPV, RK13 cells displayed an expression profile similar to that seen with HPV-11-infected HaCaT, A431, and KH-SV cells and HPV-40-infected

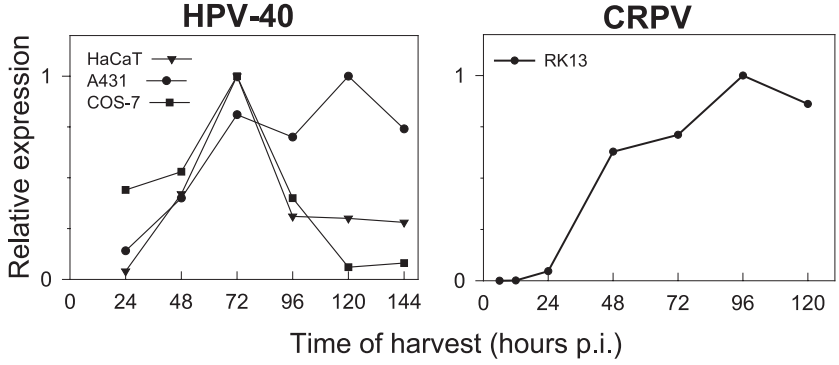

Fig. 3. Expression profiles of other papillomaviruses. HPV-40 virions were used to infect monolayers of A431, COS-7, and HaCaTs while infectious CRPV was used to infect RK-13 cells using the experimental design described in Fig. 1. Infected monolayers were harvested with TRIzol at the indicated time points and assayed by QRT-PCR.

A431 cells. These data suggest that the early events of infection with HPV-40 and CRPV occur with kinetics similar to those of HPV-11.

Adsorption to cell surface

We questioned whether the delayed expression of viral transcripts might be due to an inability of the virions to quickly adsorb to the glycocalyx of the cultured cells rather than due to the slow entry of adsorbed virus. To assay for adsorption rates using QRT-PCR, monolayers were infected with HPV-11 and rinsed at 6, 12, 24, 36, or 48 h p.i. All monolayers were harvested at 96 h p.i. Fig. 4 shows that for most cell lines, the majority of virions contributing to expression at 96 h p.i. could resist rinsing by 24 h p.i. HaCaT cells showed the highest adsorption rate with two-thirds of the contributing infectious virions being resistant to rinsing at 6 h p.i. BO-SV cells displayed a very slow adsorption rate in two independent experiments. These human keratinocytes do not express α6-integrin which was previously shown to be necessary for HPV-6b VLP binding (Evander et al., 1997), but not necessary for successful infection by BPV-4 (Sibbet et al., 2000) and HPV-11 (Kirnbauer, personal communication). It is conceivable that the poor adsorption rate of HPV-11 to BO-SV cells might be directly or indirectly influenced by the lack of α6-integrin at the cell surface, although this possibility was not tested.

Additionally, we performed experiments to determine if rinse-induced shear stress on the monolayer might, by some unknown mechanism, retard viral infection (Fig. 5). Shear stresses generated by repeated "mock" rinsing with cell supernatants at 24 h p.i. showed no effect on the final expression of E1^E4 transcripts at 96 h. This lack of effect

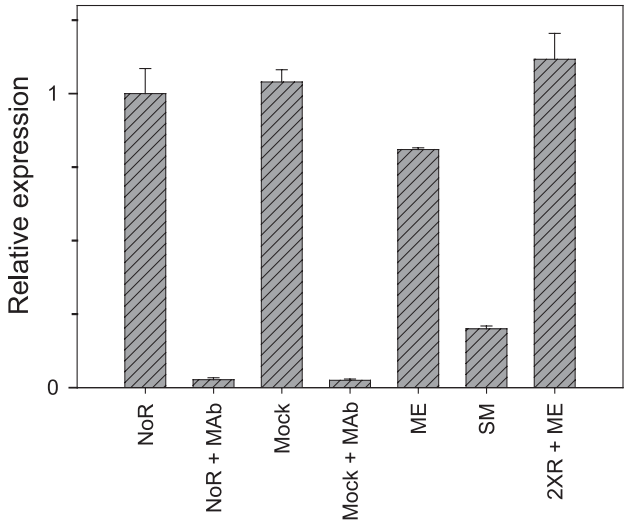

Fig. 5. Effect of rinsing on viral infection. Near confluent monolayers of A431 cells were infected with HPV-11 in 1 ml medium. Twenty-four hours postinfection, monolayers were left unrinsed (NoR), mock-rinsed with cell supernatant (Mock), medium exchanged without rinsing (ME), or rinsed 2× before a medium exchange (2×R + ME). The spent medium from ME wells was transferred onto uninfected monolayers to assay for any remaining unbound virus (SM). Additionally, H11.H3, a capsid-targeting neutralizing MAb, was added to select unrinsed monolayers (NoR + MAb) and to select mock-rinsed monolayers (Mock + MAb). At 48 h p.i., all wells were fed additional 2 ml of DMEM and cell contents were harvested at 96 h. Results shown are means (±SEM).

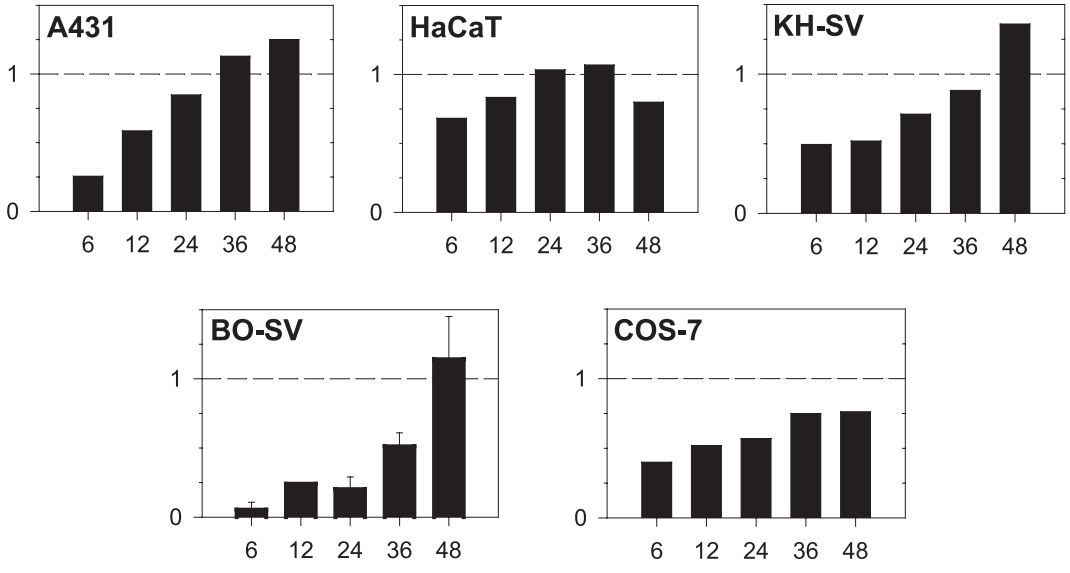

Fig. 4. Kinetics of adsorption to monolayers. Near confluent monolayers in 6-well plates were infected with 150 particles per cell of HPV-11 in 1 ml medium followed by incubation at 37 °C. At the indicated time points (hours postinfection), monolayers were rinsed 2× with PBS (abscissa). After rinsing, monolayers were given 1 ml fresh medium with an additional 2 ml medium given to all cultures at 48 h p.i. to reduce the possibility that differences in feeding would strongly affect E1^E4 expression at harvest. All monolayers were harvested with TRIzol at 96 h p.i. E1^E4 expression in each rinsed monolayer is shown on the ordinate relative to expression in an unrinsed HPV-11-infected monolayer given 2 ml additional medium at 48 h p.i. (dotted line). Adsorption of HPV-11 to BO-SV was determined by two independent experiments due to unexpected results. BO-SV results are shown as means (±SEM).

is seen both in samples treated with neutralizing MAb following the shear stress treatment and in samples without added neutralizing MAb, suggesting that neither viral expression nor post-rinse viral entry was affected by this treatment. Additionally, no significant differences were seen between monolayers receiving replacement medium versus monolayers rinsed 2× before medium replacement. Evidence of a small amount of infectious virions in the spent medium of A431 cells transferred at 24 h to an uninfected monolayer is also shown. Finally, experiments have demonstrated no difference in E1^E4 expression between monolayers rinsed with PBS versus those rinsed with DMEM (data not shown).

To determine how a 1-h incubation at 4 °C immediately following infection affects adsorption, we incubated HaCaT cells at 4 or 37 °C for 1 h following infection with HPV-11 and then placed both plates at 37 °C for five additional hours before rinsing and medium replacement. This experiment revealed that levels of E1^E4 transcripts at 48 h were not affected by a 1-h incubation at 4 °C immediately following infection (data not shown).

Viral entry

To determine if the delayed expression of E1^E4 transcripts seen in tested cell lines might be due to the slow entry of virions following their attachment to cultured cells, we measured the rate at which adsorbed virions were protected from neutralization by capsid-targeting MAbs and a nonspecific high-molecular-weight microbicide, polystyrene sulfonate (PSS) (Christensen et al., 2001). In these experiments, cells were infected with 150 virus particles per cell and incubated at 37 °C for 24 h at which time all monolayers were rinsed to remove unattached virions. At subsequent time points, a capsid-targeting MAb, or PSS, was added to neutralize any accessible virions. All cultures were then harvested at 96 h p.i. Fig. 6 shows the results of experiments with the neutralizing MAb H11.H3 (IgG2b) which targets the HI hypervariable loop of L1 in a conformationally dependent manner (Ludmerer et al., 2000). In all five cell types tested, the large majority of virions that eventually contribute to the levels of viral E1^E4 transcripts at the 96-h time point could be neutralized by the MAb at 24 h p.i. Addition of H11.H3 at 48 h p.i. neutralized a large percentage of contributing infectious virions on several cell lines and remarkably at least two cell lines showed that a significant number of virions could be neutralized even at 72 h p.i. It should be stressed that all cultures were rinsed at 24 h p.i.; therefore, virions neutralized at 48 and 72 h remained accessible and susceptible to capsid-targeting MAb for at least 24 or 48 h, respectively.

To investigate the possibility that conformational changes may occur in the viral capsid upon binding its cell surface receptor(s), similar experiments were performed with three additional MAb antibodies, H11.B2 (IgG2b) which binds the DE hypervariable loop (Ludmerer et al., 1996), G131S11.G3 (IgM) which requires hypervariable loops FG and HI (Ludmerer et al., 2000), and H6.C6 (IgG2a) which binds a linear epitope at the N-terminus (Christensen et al., 1996). We hypothesized that if large conformational changes occurred in the capsid following interaction with the cell surface, then H6.C6, which binds a linear epitope, might neutralize a larger fraction of HPV-11 virions at later time points than the three antibodies binding conformationally dependent epitopes on the hypervariable loops. We performed these experiments using three different

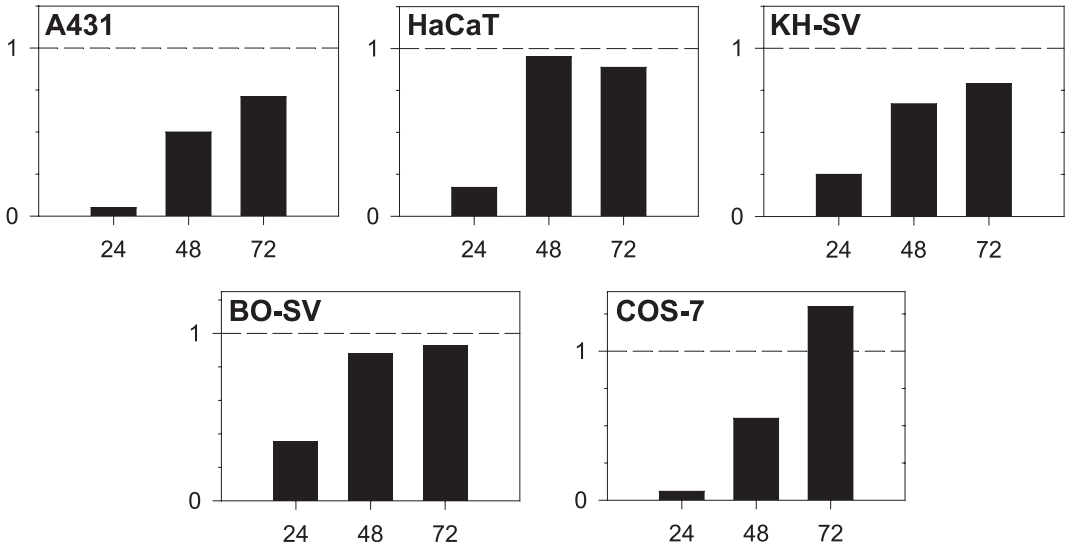

Fig. 6. Escape of HPV-11 from neutralization by capsid-targeting MAb. Near confluent monolayers were infected with HPV-11 in 1 ml medium then incubated for 24 h at 37 °C. All monolayers were then rinsed 2× with PBS followed by replacement with 3 ml medium. At the indicated time points (hours postinfection), excess H11.H3 (IgG2b) was added to cell culture supernatants (abscissa). All monolayers were harvested at 96 h p.i. E1^E4 expression in each monolayer is shown on the ordinate relative to that seen in monolayers treated with H16.V5, an HPV-16 specific IgG2b (dotted line).

cell lines but saw no differences in the ability of the various MAb to neutralize attached virus at 24, 48, and 72 h (Fig. 7a). Additionally, in a separate experiment, we tested the ability of PSS to neutralize virus adsorbed to A431 cells at the same time points postinfection and found that this nonspecific high-molecular-weight microbicide blocked infection with kinetics similar to all MAb tested (Fig. 7b). These experiments suggest that, even 24–48 h after adsorption to the cell surface, a large fraction of virions have not undergone structural changes affecting three conformationally sensitive surface epitopes.

Consistent with the findings for HPV-11, the majority of HPV-40 contributing to the 72-h expression in A431 could be neutralized at 30 h p.i. by H40.B5, a MAb prepared against HPV-40 L1 VLP in our lab (Christensen et al., unpublished data) (Fig. 7c). Similarly, greater than 50% of the CRPV contributing to 96-h expression levels in RK13 cells could be neutralized by CRPV.1A (Christensen and Kreider, 1991) added at 30 h p.i. (Fig. 7d).

Because COS-7 cells showed a unique expression profile for HPV-11 with the peak in expression of E1^E4 transcripts at 48 h, we performed an additional experiment to determine the entry kinetics of the virions contributing to this peak. The addition of H11.H3 to cell culture supernatants at 8 h p.i. completely eliminated viral transcripts at 48 h. Addition of H11.H3 to supernatants at 24 h p.i. reduced the number of viral transcripts present at 48 h by more than 4-fold from those present in cells treatment with an isotype control (data not shown). These findings indicate that the majority of virions contributing to the 48-h peak of E1^E4 expression in COS-7 cells remain both accessible and susceptible to neutralizing antibodies even 24 h p.i.

Discussion

Our experiments suggest that papillomavirus infection of cultured cells occurs with remarkably slow kinetics under the described experimental conditions. Additionally, there appears to be a large "window of neutralization" for papillomaviruses, defined as the period following adsorption during which virions are both accessible and susceptible to neutralization by capsid-targeting MAb. This large "window" is most easily explained by slow entry into the cytoplasm, but conceivably might be due to an extended period in the endosomal pathway allowing pinocytosed MAb to access virus-containing vesicles leading to neutralization within endosomes. Such extremely slow entry/trafficking using infectable cell lines is unreported for other in vitro infection models using naked or enveloped viruses. Other small, naked, DNA viruses, such as SV40 and mouse polyomavirus, have been shown to enter cells within several minutes to several hours at wide-ranging m.o.i. with no reports of a large population of virions being retained at the cell surface (Kartenbeck et al., 1989; Mackay and Consigli, 1976).

The slow expression kinetics of HPV-11 cannot be explained solely by low adsorption rates. Sufficient virions to produce measurable levels of E1^E4 transcripts in A431 cells at 96 h (by nested RT-PCR) can resist stringent rinsing only 15 min p.i. (unpublished results). For all but one cell line tested, our data demonstrate that when infected with 150 particles per cell, the majority of authentic HPV-11 virions contributing to viral transcripts at 96 h have adsorbed to the infected monolayer by 24 h p.i. Consistent with these findings, C127 cells were

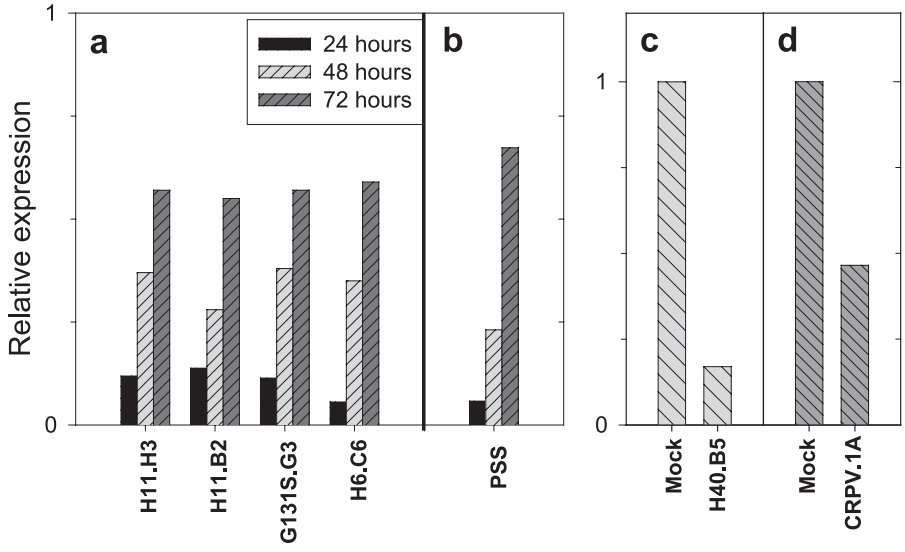

Fig. 7. Neutralization of pre-adsorbed papillomaviruses. (a and b) Comparison of abilities of neutralizing MAbs (a) and polystyrene sulfonate (PSS) (b) to inhibit infection by pre-adsorbed virions. Near confluent monolayers of A431 cells were infected with HPV-11 in 1 ml medium and then rinsed at 24 h p.i. Excess capsid-targeting MAbs or polystyrene sulfonate (100 μg) was added at the indicated time points. All monolayers were harvested at 96 h p.i. with TRIzol. Expression is shown relative to mock-treated monolayers. Similar results were obtained for the same MAb panel using COS-7 and KH-SV cells (data not shown). (c) Ability of H40.B5 to neutralize HPV-40 30 h after infection of A431 cells. (d) Neutralization of CRPV by CRPV.1A 30 h after infection of RK13 cells.

found by Müller et al. (1995) to have the highest adsorption rates for BPV-1 virions (>90% attachment within 45 min), however, we have shown that BPV-1 infection of these same cells can be largely neutralized by capsid-targeting antibodies even 24 h p.i. (Christensen et al., 1995).

Binding of HPV-11 to HaCaT cells was not improved by a 1-h incubation at 4 °C following infection. This finding correlates with the lack of benefit found for low temperature incubation on the binding of mouse polyomavirus to primary mouse embryo fibroblasts (Mackay and Consigli, 1976). The m.o.i. used in our experiments (150 particles per cell) is unlikely to saturate the ubiquitous attachment receptors (heparan sulfate) for papillomaviruses. Other groups have infected similar cell lines at much higher m.o.i. and reported no evidence of saturation (Zhou et al., 1995). Importantly, the ability to neutralize virions 24–72 h p.i. was observed following a 2× rinse at 24 h (Fig. 6). Therefore, only virions which had successfully attached to the cell surface by this time point could contribute to transcript levels at 96 h.

While it is clear from our work and the findings of others (Ozbun, 2002; Sibbet et al., 2000) that a small, yet detectable amount of virions enters and initiates transcription within a few hours postinfection, a quantitative analysis of E1^E4 transcripts shows a large increase in viral transcripts 48–120 h p.i. depending upon cell line. The ability to significantly reduce the level of viral transcripts at time points of peak expression using a capsid-targeting MAb applied hours and even days postinfection, indicates that the increase in E1^E4 viral transcripts seen over time is due neither to vDNA replication nor to increases in the rate of transcription per viral genome, but rather to the retarded entry/trafficking of previously adsorbed virions. More limited experiments with HPV-40 and CRPV confirm that other papillomaviruses enter host cells with similar slow kinetics. Our data suggest, in fact, that the majority of adsorbed virus particles remains at the cell surface for 24 h or more during which time virions are equally susceptible to nonspecific microbicides and to neutralization by capsid-targeting MAb binding either linear or conformationally sensitive epitopes. This finding argues that papillomaviruses do not undergo large conformational changes following prolonged interaction with the cell surface, although subtle changes in capsid structure cannot be ruled out.

Retarded entry kinetics might conceivably be due to multiple causes including sparse or occluded secondary receptors, a clustering requirement before endocytosis, or complex interactions with primary receptors. Alternatively, papillomavirus virions might be endocytosed and recycled to the cell surface multiple times before being properly trafficked to the cell nucleus. Secondary receptors for papillomaviruses are currently only hypothetical. The fact that one virion per cell can lead to a detectable in vitro infection (Culp and Christensen, 2003; Ozbun, 2002) argues against the need to cluster virions and suggests that papillomaviruses are highly efficient in their ability to interact with endocytic machinery to insure appropriate targeting. We are currently investigating the hypothesis that excessive interactions with heparan sulfate moieties at the cell surface impede viroplexis.

It is likely that papillomavirus particles might be capable of entering cells by both infectious and noninfectious pathways. The particular pathway used by a given particle may be determined by several factors including m.o.i., site of initial attachment, condition of the glycocalyx, and the particular characteristics of a given particle. It has been shown in the mouse polyomavirus system, for example, that bulk internalization of pseudovirions can occur by a receptor-independent, cytochalasin D-sensitive pathway which is separate from the receptor-dependent, cytochalasin D-insensitive endocytosis utilized by individual polyomavirus virions and pseudovirions that succeed in delivering DNA for transcription (Krauzewicz et al., 2000).

It remains unclear how accurately surrogate papillomavirus particles such as VLPs and pseudovirions model the uptake of authentic virions. Recently, it was reported that HPV-16 VLPs and BPV-1 virions applied at a high m.o.i. (1.5×10^4) co-localized within vesicles in C127 cells at 4 and 8 h p.i. (Day et al., 2003). It is difficult to know, however, whether the population of BPV-1 virions shown to be co-localizing with the VLPs was progressing successfully down an infectious pathway. Because of the limits of detection, studies that rely primarily upon fluorescent microscopy or EM for determining papillomavirus entry kinetics may have difficulty in distinguishing between an infectious and a noninfectious pathway of entry. Because QRT-PCR measures a product of successfully targeted vDNA, the experiments we have described reveal only the kinetics of the infectious pathway.

For the studies described in this report, cells were infected with approximately 150 particles per cell as determined by an EM count of particles in virus stock. The number of actual infectious virions is presumably lower, although this was not empirically addressed. While an m.o.i. of 150 would be considered high for many viral systems, it is considerably lower than that used in previous studies on BPV-1 entry (Day et al., 2003; Liu et al., 2001; Zhou et al., 1995). Papillomaviruses can initiate in vitro infections at an m.o.i. of <1 (Culp and Christensen, 2003; Ozbun, 2002), but the level of transcripts generated at such low m.o.i. is below the sensitivity level of our QRT-PCR assay.

It was previously reported that monolayers grown to higher densities showed a reduced rate of internalization for BPV-1 virions (10^6 particles per cell) as assayed by EM (Zhou et al., 1995). It was suggested by Zhou et al. that the ability to neutralize a large fraction of BPV-1 virions 24 h following infection (Christensen et al., 1995) might be due to the use of cells at higher density than those used in EM studies. While a correlation between slow entry rate and high cell density cannot be ruled out for every cell line–virus combination, our observations with HPV-11 and multiple cell

lines are contrary to this, suggesting rather that confluent monolayers take up virions faster than subconfluent cells (Christensen et al., 1995, and unpublished results). While natural infections cannot be perfectly modeled using cultured cells, it seems reasonable that the infection of near confluent cells more closely models in vivo infections than infection of cells at low density.

Interestingly, raw Ct values suggest that the two human keratinocyte cell lines transformed with SV40 had relatively poor expression of HPV-11 E1^E4 transcripts (Table 1). Poor HPV-11 expression cannot be due to SV40 transformation alone as COS-7 cells showed strong expression for both HPVs tested. It is notable that COS-7 cells, the least appropriate host cell for HPV infection in this study, seem to have different entry/expression kinetics for HPV-11 than the more biologically relevant human keratinocytes and epithelial cells.

Importantly, rabbit keratinocytes infected with CRPV showed similar kinetics to those seen in human keratinocytes infected with HPVs. In as far as the infection results for cultured keratinocytes and epithelial cells reflect the in vivo infection kinetics of damaged epithelium, our findings suggest that capsid-targeting circulating and mucosal antibodies may have a large window of opportunity to access and block infection following the introduction of papillomaviruses to the epithelium.

Materials and methods

Cells and virus

A431 and COS-7 cells were grown in DMEM (5% FBS). HaCaT cells (Boukamp et al., 1988) were grown in DMEM (10% FBS). RK13 cells were grown in Eagle's medium (10% FBS). BO-SV and KH-SV were cultured in KGM (Clonetics). HPV-11 stocks were produced and quantified as previously described (Culp and Christensen, 2003). Briefly, HPV-11-infected xenografts were harvested from athymic mice and homogenized to release virus. HPV-11 particles were quantified by EM. CRPV virions were obtained from infected rabbit xenografts grown in athymic mice. HPV-40 was obtained from a patient lesion as previously described (Christensen et al., 1997).

Infections/neutralization

Cultured cells were trypsinized and seeded at 5×10^5 (A431, COS-7, HaCaT, and RK13) or 1×10^6 (KH-SV and BO-SV) in 6-well plates or 35-mm culture dishes and infected 24–48 h later when 90–95% confluent. Spent medium was removed and replaced with 1 ml fresh medium containing virus for the infection step. Where indicated, rinsing was performed by aspiration of cell culture supernatant followed by two rinses with 3 ml PBS each. For neutralization experiments shown in Fig. 6, H11.H3 and H16.V5 were each used at a final concentration of 133 nM. For the HPV-11 neutralization experiments shown in Figs. 5 and 7, all final concentrations of antibodies were 4× the titered concentration required for neutralization, specifically 1 nM (H11.H3), 16.7 nM (H11.B2), 4.4 nM (G131S.G3), and 17.1 μM (H6.C6). Adsorbed CRPV was neutralized with a 1:1000 dilution of CRPV.1A ascites (in DMEM). Adsorbed HPV-40 was neutralized with a 1:200 dilution of H40.B5 ascites (in DMEM). For all experiments, at the indicated time points postinfection, cells were lysed and harvested with TRIzol (Invitrogen).

Assay of viral transcripts

Total RNAs were isolated from TRIzol by alcohol precipitation and RNA concentration was determined by spectrophotometry. QRT-PCR was performed as previously described with the levels of E1^E4 transcripts compared based on the levels of the endogenous reference transcript (TBP) (Culp and Christensen, 2003). One microgram of total RNA was assayed in triplicate multiplex reactions for the levels of TBP and E1^E4 transcripts using Quantitect Probe RT-PCR Kit (Qiagen) on the Mx-4000 (Stratagene). Amplification primers (Penn State College of Medicine Core Facility) and Taqman fluorogenic probes (Biosearch Technologies) for TBP and HPV-11 E1^E4 were used as previously described (Culp and Christensen, 2003). Amplification primers for HPV-40 were 5′ TGTGCAGCTACAGAGTGACA 3′ and 5′ GTCAGCAGTCTCAACAATGG 3′ and were used at 400 nM and 1.2 μM, respectively.

The fluorogenic probe for HPV-40 was 5′ 6-FAM d(TGGCAGACTCTCCAGTACTATCGAGGAA) BHQ-1 3′ (Biosearch Technologies) used at 100 nM. CRPV E1^E4 transcripts were amplified using 5′ GTGCCCGGAGTGTTGTAA 3′ and 5′ GGTGTCTTCAGGGGCACT 3′ used at 400 nM and 1.2 μM, respectively. The fluorogenic probe specific for CRPV transcripts was 5′ 6-FAM d(TGAAAATGGCTGAAGCTCCCC) BHQ-1 3′ (Biosearch Technologies) and was used at 100 nM. All QRT-PCR data were analyzed using REST software (Pfaffl et al., 2002).

Nested RT-PCR for HPV-11 E1^E4 containing transcripts was performed as previously described (Ludmerer et al., 2000) using the GeneAmp RNA PCR Core Kit (Applied Biosystems).

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